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Table of Contents

Introduction: Most of the likely agents of bio-terrorism have profound effects on the host and, in particular, on the immune and inflammatory responses. For many of these agents, pathogenesis has been studied at the cellular and molecular levels. These studies indicate that each specific organism has distinctive effects on the host immune and inflammatory cells that contributes to the unique clinical characteristics of the disease. These studies largely have focused on how the agent and its toxins and other constituents modulate host cell expression of individual cytokines and other molecules of interest as well as activation pathways. We have proposed a broad-based approach to identify the unique "signatures" of infectious agents using host DNA micro-arrays. Because of the known diverse patterns of host cell interactions with these organisms, examination of the host transcriptional response has enormous potential to allow rapid diagnosis of infectious diseases in general and agents of bio-terrorism in particular. The high through-put and scale of analysis possible with the use of DNA micro-array technology offers an ideal platform for these studies allowing us to identify and focus on a group of host genes that are most informative in this regard. Key signature genes will serve as the basis for rapid diagnostic approaches that could be accessed when an outbreak is suspected. Thus, recognition of specific genes that are expressed or repressed during these diseases will provide signature markers that can be used in related and alternative approaches for rapid diagnosis. *In the present report, section ONE, we present our most recent results of this project and describe the definition of genesets which define infection by select agents in man, which we term "infection signatures".*

The immune response to infection varies between individuals. This variation is genetically defined and can be examined through the use of single point genetic polymorphisms in the individual (single nucleotide polymorphisms – SNPs). Broadly speaking, it is possible to use such genetic information to determine whether an individual, or group of individuals, is more or less susceptible to infection or whether such an infection is likely to be more or less severe. In fact, it is possible to use genetic markers to determine whether individuals are likely to have severe, life-threatening infection or not. We consider that such information may be of use in determining whether servicemen and women, or members of the intelligence services should be sent into certain biothreat theaters. *In the present report, section TWO, we present our preliminary analysis of immunologically relevant genetic markers; that is, markers which define higher or lower production of essential immunomodulatory materials: cytokines.*

In our previous report we presented important microarray data for the select agent *F.tularansis* following experimental infection of rodents. In the current year, we have carried this work forward to explore early detection of *F.tularansis* by searching for antibodies in saliva. *In the present report, section THREE, we present our initial data showing that specific antibodies can be detected in saliva.*

Finally, mindful of the fact that in addition to detection of select agents, novel therapeutic strategies are required, we have conducted a pilot experiment in this area. Humans and other species have a wide range of naturally-occuring innate immune response mechanisms, known collectively as 'anti-microbial peptides'. One of these, LL37 has proven useful in the destruction of vaccinia viruses. We tested its efficacy against the select agent *B.anthracis*, and noted that it was capable of killing this organism. *In the present report, section FOUR - APPENDIX, we present our data showing the killing of B.anthracis by LL-37 and suggesting the importance of the bacterial capsule in LL-37 resistance.*

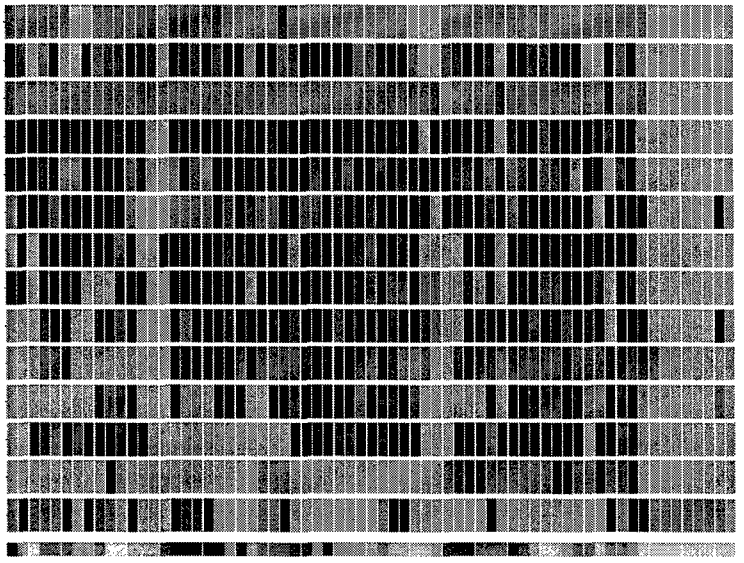
SECTION ONE – A BACTERIAL INFECTIONS

Exposure to biological agents can be potentially monitored in many different ways. The majority of current identification techniques rely on detection of the organisms themselves, through culturing or identification of genetic or proteinaceous components. Patients may also be monitored through identification of circulating antibodies via ELISA or other means. Culture of the organisms can be time consuming, and small amount of infectious material may be difficult to sample in many instances. Similarly, antibodies take several days to appear and are not an efficient way to measure exposure.

The whole blood infections described in these studies offer an efficient way to measure exposure to any number of biological agents. By evaluating the host immune response to these pathogens via analysis of mRNA, we are able to avoid the pitfalls of other detection techniques. Least of all is the availability of starting material. While it may be difficult to recover infectious particles from a patient with a low level of exposure, blood samples can easily be taken from a large number of potential victims. By identifying genes that are regulated exclusively upon contact with certain agents, we have taken advantage of one of the most stringent and disciplined detection systems available; the human immune response.

From a research perspective, microarray data of this nature gathers much more than selectivity lists for restricted agents. Each set of infections brings with them a wellspring of knowledge about the immune response as a whole. Knowing which genes are up or downregulated is not only a valuable diagnostic tool, but provides important insight into which cell types are involved in the pathogenesis of disease.

Early symptoms arising from attack by, or infection with pathogens from the A- and B-list of potential bioweapons are extremely difficult to distinguish from those due to minor, everyday infections. Furthermore, identifying attackers who are purposefully infected in order to carry disease into the United States is profoundly difficult when early-stage infection symptoms are so readily confused. Finally, traditional microbiological or nucleic acid-based diagnostic approaches are limited in sensitivity by the availability of sufficient numbers of organisms within the infected individual; approaches often possible only after that individual has become infectious to others or has progressed beyond the point of therapeutic intervention Our approach in previous funding periods has been to identify key infection signatures of host gene expression in response to infection with bioweapon pathogens. We have now successfully identified "infection signatures" of host genes which indicate the presence of live bacterial bioweapons, *B. anthracis, Y. pestis F. tularensis, M. tuberculosis* (multi-drug resistant) and *B. mallei.*To illustrate this point, the gene expression picture which defines the infection signature for antrhax infection is shown below. Similar pictures for plague, glanders and MDR tuberculosis are available.



INFECTION SIGNATURE FOR ANTHRAX *- - A consideration is made as to whether genes are turned on (red) or off (green), following infection with anthrax, plague, glanders or MDR-TB. There is a strong clustering of anthrax response patterns at the right hand side of the slide (anthrax indicated in yellow, bottom row) while those for others are distributed more randomly (plague, navy; glanders, blue; MDR-TB, red). This pattern of thirteen genes "off" and one "on" represents the "**infection signature**" for anthrax and is the basis for the work proposed in this subproject. Other organisms have infection signatures which use different genes from each other, enabling great specificity to be obtained.*

Funding in future current periods will bring these infection signatures closer to use as a deployable device, with potential use in the field or to prevent border incursion. We will design and test a custom detection device of microarray format. This device will contain three sets of key genes: host response (infection signature) genes and bioweapon-specific genes (identifying *B. anthracis* etc specifically) will enable us to detect known pathogen threats from the A- and B-list select agents. In addition, genus-specific ribosomal RNA genes and genes encoding virulence factors and toxins will extend our capacity to include detection of novel, genetically-manufactured agents (such as those recognized as having been produced in the past by the insertion of the botulinum toxin gene into *Y. pestis*).

The tables below illustrate the selectivity process applied to microarray data for each organism. RNA expression profiles were analyzed using GeneSpring software to generate hierarchical clustering maps, which organize experimental groups based on their expression of selective genes. Specifics on the process of gene selection are available on request. Table 1 contains the numbers of genes whose expressions patterns change upon stimulation with the indicated agent. This list is trimmed upon analysis via the GeneSpring software, and the final *selectivity* lists are generated. The *exclusive selectivity* tables are generated upon removal of any genes that appear in more than one *selectivity* list. The exclusive selectivity genes appear in Table 2.

Table 1

	3 hour incubation		8 hour incubation	
ORGANISM	**up regulated**	**Down regulated**	**up regulated**	**down regulated**
Bacillus anthracis	17	144	119	80
Burkholderia mallei	226	33	286	336
Yersinia pestis	442	67	212	373

	Virulent		Avirulent	
ORGANISM	**up regulated**	**down regulated**	**up regulated**	**down regulated**
Bacillus anthracis	1	17	-	2
Burkholderia mallei	3	1	6	-
Yersinia pestis	9	-	N/A	N/A

Table 2

Anthrax Virulent	**Plague Virulent**	**Glanders Virulent**
general transcription factor II, i	tight junction protein 2 (zona occludens 2)	properdin P factor, complement
enolase 2, (gamma, neuronal)	deafness, autosomal dominant 5	CD164 antigen, sialomucin
early growth response 1	growth arrest and DNA-damage-inducible, alpha	hypothetical protein FLJ20637
splicing factor, arginine/serine-rich 4	synaptotagmin V	KIAA1466 protein
hypothetical protein MGC5139	laminin, beta 3	
fucosidase, alpha-L- 1, tissue	mitogen-activated protein kinase 13	
popeye domain containing 2	phospholipase A2, group IVA (cytosolic, calcium-dependent)	
E3 ubiquitin ligase SMURF2	GM2 ganglioside activator protein	
recombining binding protein suppressor of hairless (Drosophila)	Homo sapiens transcribed sequence with moderate similarity to protein ref:NP_062553.1	
POP7 (processing of precursor, S. cerevisiae) homolog		
KIAA0265 protein		
KIAA0853 protein		
v-myb myeloblastosis viral oncogene homolog (avian)-like 1		
neuroendocrine differentiation factor		
serologically defined colon cancer antigen 1		
Notch homolog 1, translocation-associated (Drosophila)		
hypothetical protein FLJ20847		
hypothetical protein FLJ20094		

Table 2 cont'd.

Anthrax Avirulent	Plague Avirulent	Glanders Avirulent
advillin	chondroitin sulfate proteoglycan 2 (versican)	three prime repair exonuclease 1
adenosine A1 receptor	suppressor of cytokine signaling 1 /// suppressor of cytokine signaling 1	colony stimulating factor 3 (granulocyte)
	metallothionein 1H	interferon-induced protein 35
		tripartite motif-containing 5
		19A24 protein
		GTP binding protein 1

The hierarchical cluster (gene tree) images for each agent are available. Further work on this topic is directed towards validation of the lists by quantitative PCR.

Bacterial Infection-Ongoing Studies

At this point, the microarray data generated from these infections can be analyzed using additional software packages. One such software package, Pathway Assist, uncovers common metabolic pathways and direct interactions shared by seemingly unrelated genes. Rather than focusing on several genes at a time, this software offers a glimpse at a more global picture and can reveal critical interactions that might otherwise have gone unnoticed. We are currently using this tool to analyze each of the gene lists above.

Not only does this software find common cellular pathways and interactions between proteins of interest, but can screen genes of interest against components of known pathways such as apoptosis and cell proliferation. The figure below is an illustration of how expression data from fits into a cell proliferation pathway. The data in this figure comes from a virulent plague sample. When manipulating the data, one can select the lines drawn between molecules of interest. These lines represent correlations that have been reported in manuscripts listed on Pubmed, which can be viewed directly by clicking on the image. lines drawn between molecules a pathway list generated with the selectivity genes from virulent Anthrax infections.

Figure 1

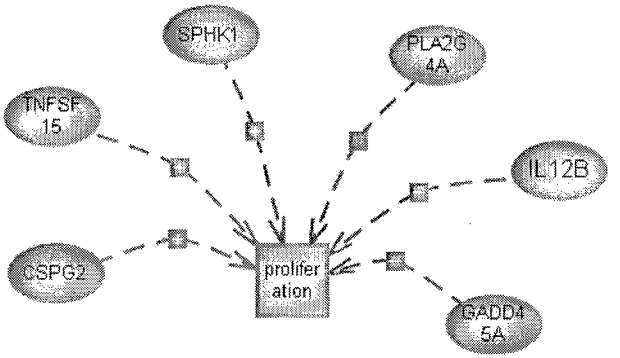
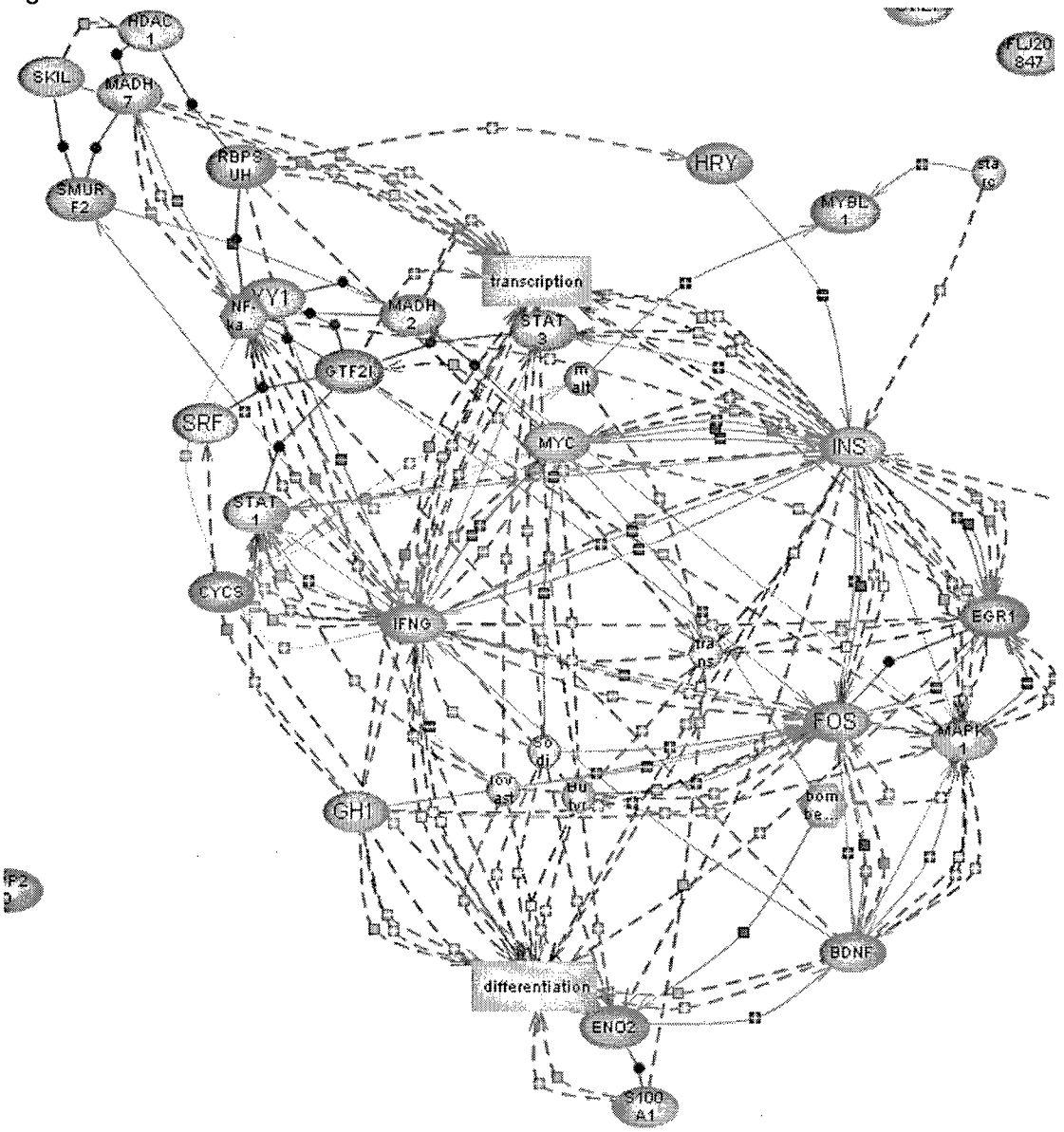


Figure 2 is an expanded version of the above figure that correlates targets from the exclusive selectivity list of virulent anthrax to various cellular processes.

Figure 2



SECTION ONE-B – VIRAL INFECTIONS

Status of Viral Whole Blood Infections

The infections involving SARS virus were completed as of October 2004 and the RNA processing for Affymetrix is ongoing for these samples. The viral infections were performed in whole blood obtained from healthy human volunteers. The blood was diluted 1:1 with RPMI 1640 and infected at an MOI of 1 (as per the preliminary studies outlined above). The blood was incubated at 37°C with 5% CO2 for 3 and 8 hours. At each time point, the blood was centrifuged to separate the cellular blood components. The white blood cell layer was removed, placed in RNAlater, and stored in -20°C. The supernatant was harvested and stored at -20°C for future studies. On the day of RNA isolation, the tube containing the white blood cells plus RNAlater was centrifuged and the RNAlater removed and discarded. From this point, the protocol for the Ribopure Blood RNA Isolation Kit was followed according to manufacturer's instructions. In addition, as required by Affymetrix, an RNA cleanup procedure using the Qiagen Rneasy kit was performed. The RNA was then transported to the Center for Applied Genomics, where it was processed on the Affymetrix Human U133 chip.

The infections involving the remaining viruses are now commencing. Influenza, Dengue and Haantan viruses have been grown and stored. Unfortunately, a critical Dengue Virus strains obtained from ATCC was found to be a contaminated stock vial. This led to the requisite reapplication for a transfer permit and significant delays in procuring the virus.

There were initial difficulties in quantifying the Dengue and Influenza viruses, due mainly to the variable ability of these agents to form visible plaques in cell monolayers. Since numerous viruses were to be investigated in these studies, attempts were made to quantify all strains in this manner.

Preliminary titers for these viruses have been obtained, but we have endeavored to ensure the accuracy of these results. Immunofluorescence-based plaque reduction assays provide enhanced plaque visualization and more reliable quantification of viral stocks. We are currently working to optimize this protocol for titration of the last virus strains. This assay will be useful in quantifying the Dengue, Hantaan and Influenza viruses.

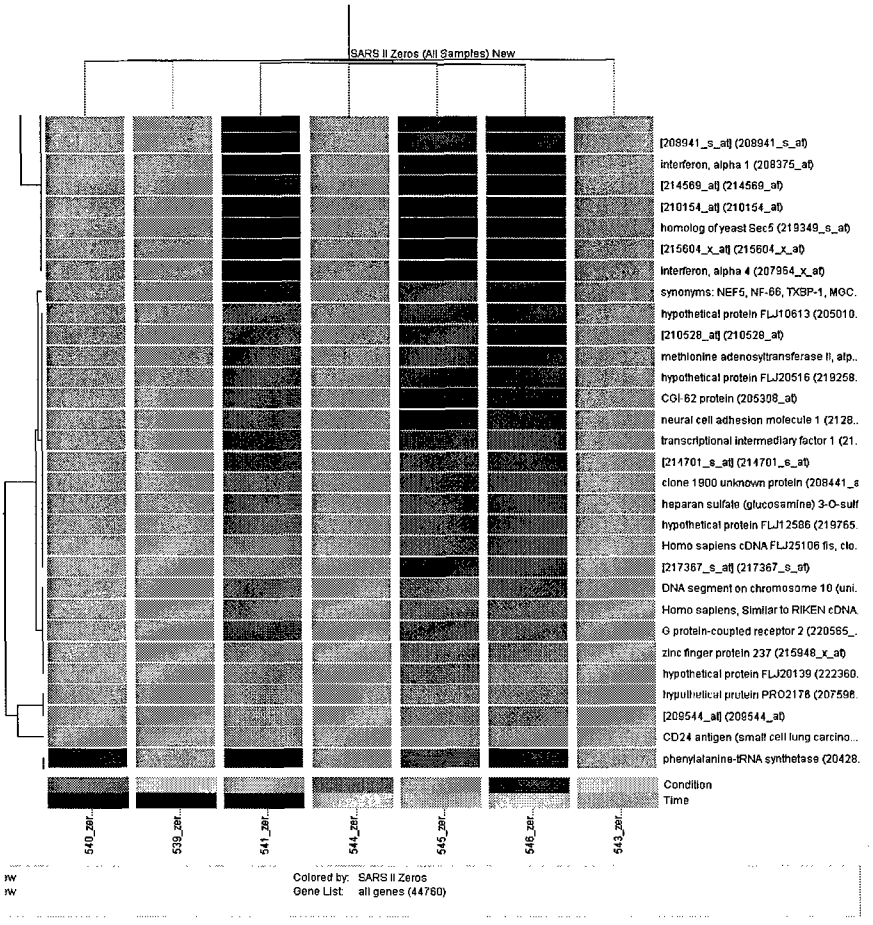
We have not yet begun studies involving Monkeypox virus. Acquiring the virus has been time-consuming process, however we have recently secured a shipping agreement from the Select Agent Distribution Activity. The paperwork for this transfer is in process and we are currently awaiting review from the Legal Department at UMDNJ.

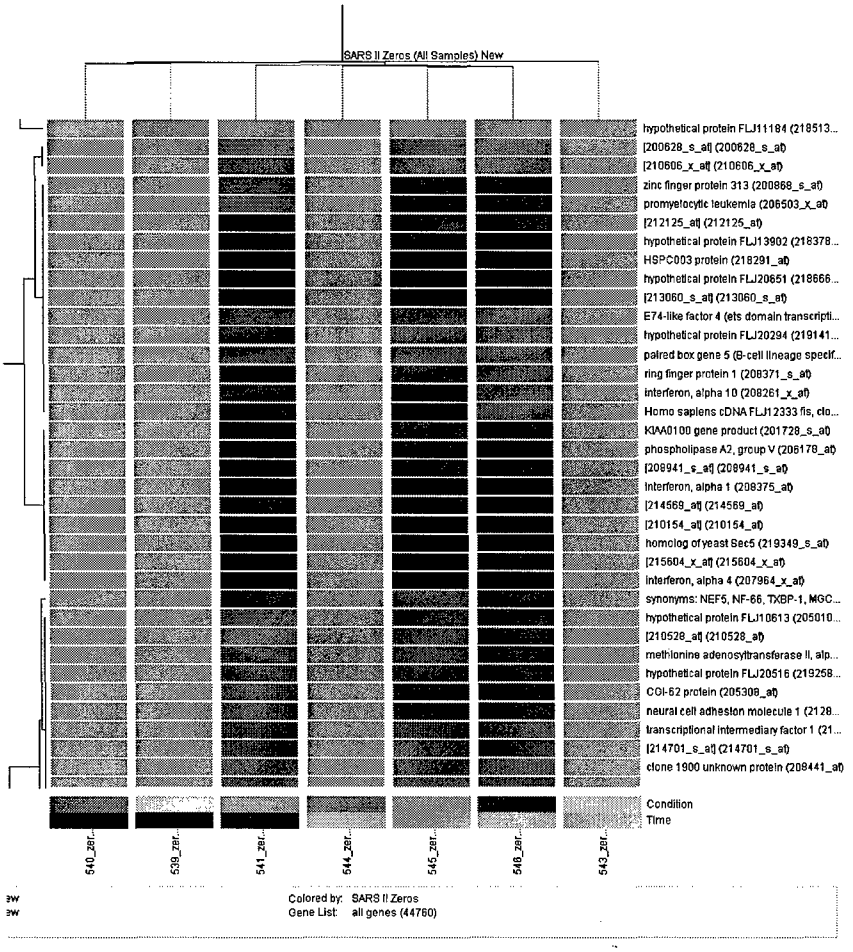
Preliminary experiments – SARS

Preliminary infections were performed to determine the appropriate amount of SARS virus to use as the inoculum for the whole blood infections. Potential differences in gene expression were investigated by stimulating whole blood cultures with three different multiplicities of infection (MOI) of virus (given as plaque forming units (PFU) per peripheral blood mononuclear cell (PBMC)). Whole blood from healthy volunteers was inoculated with MOI of 0.1, 1 or 5 and incubated at 37°C in 5%CO_2 for 3 or 8 hours. At

the appropriate timepoint, RNA and supernatants were harvested and stored for analysis. RNA was processed by the Center for Applied Genomics and evaluated as previously described using the Affymetrix Human U133 Chip. The gene tree images on the following page are from one such preliminary experiment. The bottom row of colored bars denotes time (3 or 8 hours) while the second to last represents experimental condition (yellow=uninfected, red=MOI 0.1, light blue=MOI 1 and dark blue=MOI 5). The data suggest that expression patterns of PBMC infected at MOI of 0.1 are comparable to that of uninfected groups. Likewise, samples infected at either MOI of 1 or 5 exhibit parallel RNA expression profiles. By choosing to perform the SARS infections at an MOI of 1, it is possible to investigate biologically relevant levels of viremia while preventing excess waste of valuable virus stock.

In addition to optimizing the amounts of virus used for stimulation, these experiments have revealed several genes of interest. Among these are several Interferon-alpha (IFN-a) subtypes, including 1, 4, 7, 10 and 14, which appear to be upregulated upon stimulation with SARS CoV. While it is not surprising that SARS induces IFN-a, the involvement of these particular subtypes suggest involvement of specialized antigen presenting cells known as plasmacytoid dendritic cells. This population is also known as the natural interferon-producing cell, and displays anti-viral activity to a variety of viruses, but its involvement in SARS pathogenesis has yet to be demonstrated.





SARS Inactivation Experiments

Performing an experiment, or set of experiments, solely in a BSL3 laboratory is not always plausible because of space and equipment issues. Samples contaminated with bacterial agents can be sterilized by heating, filtration or other methods and removed from the BSL3 for evaluation in a BSL2 environment. Ultraviolet light has been used to effectively to inactivate viruses and render them replication deficient. In order to determine if samples infected with the SARS virus could be safely removed from the BSL3 after UV-irradiation, SARS virus stocks and supernatants from SARS-infected whole blood were irradiated with ultraviolet light for various lengths of time and assayed for the ability to replicate in permissive VERO cells. In VERO cells incubated with SARS-infected supernatants derived from the whole blood infections described above. Supernatants were irradiated for 15, 30 or 45 minutes with germicidal UV-C (254nm) light. Infectious virus was detectable after 15 minutes of UV irradiation. However, no cytopathic effect (CPE) was detected after 45 minutes of UV irradiation. This was repeated using highly concentrated viral stocks to inoculate the monolayers, and still, no replication competent virus was detected after 45 minutes of UV-irradiation (not shown). All samples were evaluated at days 1-6 post-exposure, with CPE typically appearing within 1-2. This experiment was performed in quadruplicate for each supernatant tested, with four supernatants being tested. WE therefore believe that we have developed a simple, straightforward prorocol for inactivating the SARS virus in cell supernatants.

SECTION TWO

Genetic variation in host cytokine genes.

The human immune system is designed to recognize and fight infection from bacteria, viruses and other agents. The range of potential disease-causing organisms varies widely across the world and those present in one geographic region are often absent from another; for example, individuals from Northern Europe have not evolved an immune system with specific adaptations to deal with malaria. In general then, races and peoples developing in various parts of the world have evolved immune systems designed to protect them from relevant (ie, local) infectious agents and this has led to areas of "immune selectivity" whose geographic origins are reflected in the modern-day diverse national or continental ancestry of the citizens of the United States. It is now clear that this variation is inherited; that is, it is genetically controlled.

In 1988 Sorensen and colleagues demonstrated that adopted individuals carried a risk of death from infectious causes that was equivalent to that of their natural, rather than adoptive, parents. Thus, they established unequivocally that premature death in adults from infectious causes had a strong genetic background. Mouse studies have shown conclusively that differences in the production of cytokines is important in determining the course and outcome of infectious disease. Studies in human infection have supported this. These are perhaps best demonstrated by studies that showed that low TNF production was associated with a ten-fold risk of fatal outcome from meninococcal meningitis, while high IL-10 levels were associated with a twenty-fold risk of fatality. These two factors were independent, that is the influence of one cytokine on disease outcome was not dependent upon that of the other. These studies complimented earlier reports of high IL-10 levels being associated with fatal bacterial infections in man. Recently, IL-10 has also been shown to affect macrophage responses during mycobacterial infections. Furthermore, the severity to which meningitis progresses is associated with serum IL-10 levels, such that high serum IL-10 was observed in patients with a poor or fatal outcome, while patients who had mild disease and a good prognosis had lower serum IL-10 levels.

This phenomenon of immune-genetic diversity has two main impact points in the context of biological warfare or bioterrorism. Through their genetic makeup, serving military personnel may be at a greater or lesser risk if exposed to bio-aggression and in addition may or may not develop protective immunity if vaccinated against such agents. In this context it would be of interest to understand this phenomenon in order to perhaps select personnel who could be sent safely to a bio-warefare theatre and/or used to provide relief in the event of an attack on a US population centre. In addition there is the possible danger that the natural differences between individuals and ethnic groups within the United States population might be exploited by outside aggressors in order to attack selectively, or spare selectively, members of one or more ethnic groups. It is noteable however, that while genetic diversity in the MHC molecules (above) is widely-recognised and defined, the extent of genetic diversity in cytokine genes and the functional consequences regarding sensitivity or resistance of individuals of particular national or ethnic origins to debilitating or lethal infection, is largely unknown, since previous experiments have largely been carried out only in Northern European Caucasians. Our experiments are designed to fill this gap in our knowledge.

We have prioritized the following genes (and associated functional loci) as being particularly important in immunity against infectious diseases, both bacterial and viral:

IL-1β/-31	IL-13/-1112
IL-1β/-511	IL-13/+2044
IL-1β/+3954	IL-18/-607
IL-1β/5810	IL-18/-137
IL-2/-631	IL-6
IL-2/-330	IL-8
IL-2/+114	INFγ

Here, we present our initial data on the IL-2 and IL-13 genes, comparing the allelic distribution of these key immunoregulatory genes between Caucasian individuals and those of African descent.

Gene Locus	Study Population	Allele	Frequency
IL2, +114	Caucasian	G/G	0.50
		G/A	0.38
		A/A	0.11
	African American	G/G	0.79
		G/A	0.19
		A/A	0.02
IL-13, +2044	Caucasian	G/G	0.57
		G/A	0.43
		A/A	0.00
	African American	G/G	0.78
		G/A	0.20
		A/A	0.02

Thus, these data show significant differences in the distribution of IL-2 and IL-13 alleles between Caucasian Americans and African Americans. The functional consequence of this remains to be fully defined. However, it is known that the IL-2 +144 "A" allele is associated with higher IL-2 production. Similarly, the IL-13 +2044 "G" allele is associated with greater production of IL-13. These data lay the groundwork for future experiments where alleles which are protective against bacterial or viral agents and infections, can be defined.

We have been particularly interested in the gene interleukin-1 (IL-1) because this gene is important in inflammatory responses and it has many loci which have been associated with a range of autoimmune and inflammatory disorders. We examined the allele distribution of several markers within the IL-1 gene cluster.

The first gene to be looked at in detail IL-1β: Interleukin-1β, produced mainly by blood monocytes, mediates the panoply of host reactions collectively known as acute phase response. The multiple biologic activities that define IL1 are properties of a 15- to 18-kD protein that is derived from a 30- to 35-kD precursor. It has been that the C-to-T transition at position -31, creates a TATA box. IL-1 represents a family of polypeptides with a wide range of biological activities. At least two dissimilar gene products have been cloned; there are probably more. The human IL-1 family plays an important role in the

pathogenesis of many diseases and functions as a key mediator of the host response to various infectious, inflammatory, and immunologic challenges. SNP variations in this gene may very well affect the outcome of vaccination efficacy.

Methods: The SNPs were analyzed with either PCR amplification and RFLP analysis or ARMS-PCR. All amplified and digested samples were run on a 3% agarose gel with Denville 1kb ladder. The Denville PCR kit was used for all reactions and the protocol for IL-1β/+3954 and IL-1β/-511 was 1.5 mM $MgCl_2$, 1x NH_4 Reaction Buffer, 0.2 mM dNTP, 1 unit Taq polymerase, 0.2 μM of forward and reverse primer each and 50ng DNA from either the Caucasian or African-American normal populations, finally adding dH_20 to a volume of 25 μl. The PCR program for the same SNPs was 94°C for 4min, (94°C for 30s, 55°C for 30s, 72°C for 45s) x 36 cycles and 72°C for 5min. The protocol and program for the multiplex PCR reaction IL-1β/-31 and IL-1β/5810 was the identical but with 5 μM of each of the four primers.

Results:

SUMMARY

	IL-1β/-511		**IL-1β/-31**		**IL-1β/5810**		**IL-1β/+3954**	
Ethnicity	WoS	AAC	WoS	AAC	WoS	AAC	WoS	AAC
n	**133**	**105**	**103**	**57**	**90**	**31**	**144**	**116**
Observed	CC=60(45)	CC=22(21)	CC=14(13)	CC=26(46)	AA=13(14)	GG=13(42)	CC=97(67)	CC=92(79)
	CT=57(43)	CT=49(47)	CT=47(46)	CT=24(42)	GA=36(40)	AG=16(52)	CT=38(26)	CT=21(18)
	TT=16(12)	TT=34(32)	TT=42(41)	TT=7(12)	GG=41(46)	AA=2(6)	TT=9(6)	TT=3(3)
Expected	CC=58.9(44)	CC=20.6(20)	CC=14(14)	CC=25(44)	AA=11(12)	GG=14(45)	CC=93.4(65)	CC=90.6(78)
	CT=59.2(45)	CT=51.8(49)	CT=48(47)	CT=25(44)	AG=41(46)	AG=14(45)	CT=45.1(31)	CT=23.9(21)
	TT=14.9(11)	TT=32.6(31)	TT=42(41)	TT=6(11)	GG=39(43)	AA=3(10)	TT=5.4(4)	TT=1.6(1)
X2-value	0.093	0.155	5.100	0.078	4.860	0.546	1.550	0.640

Tabel 1: The percentage of allele frequencies is shown in parenthesis.

IL-1β/-511

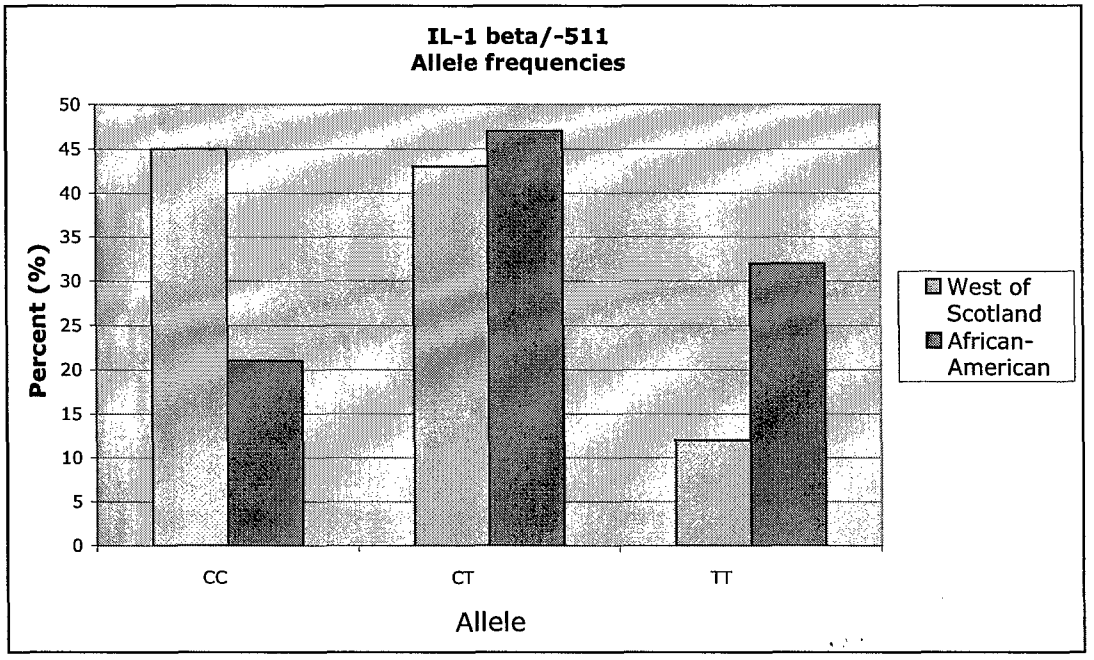


Chart 1 Comparison of the observed allele frequencies between the West of Scotland and African American normal populations for IL-1β/-511 SNP.

IL-1β/+3954

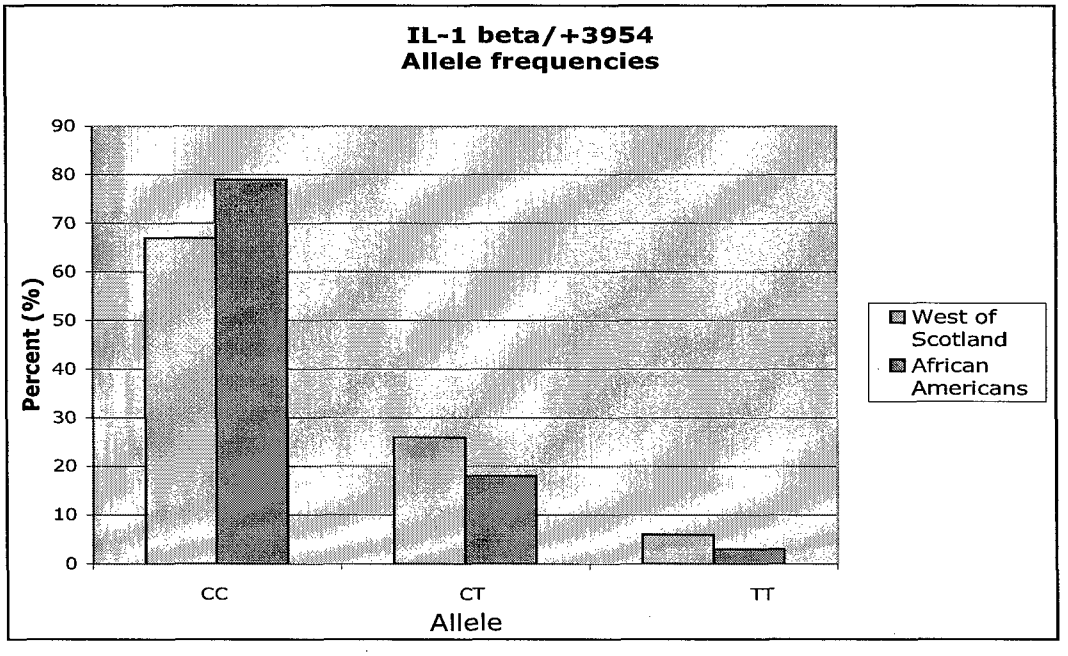


Chart 2

IL-1β/-31

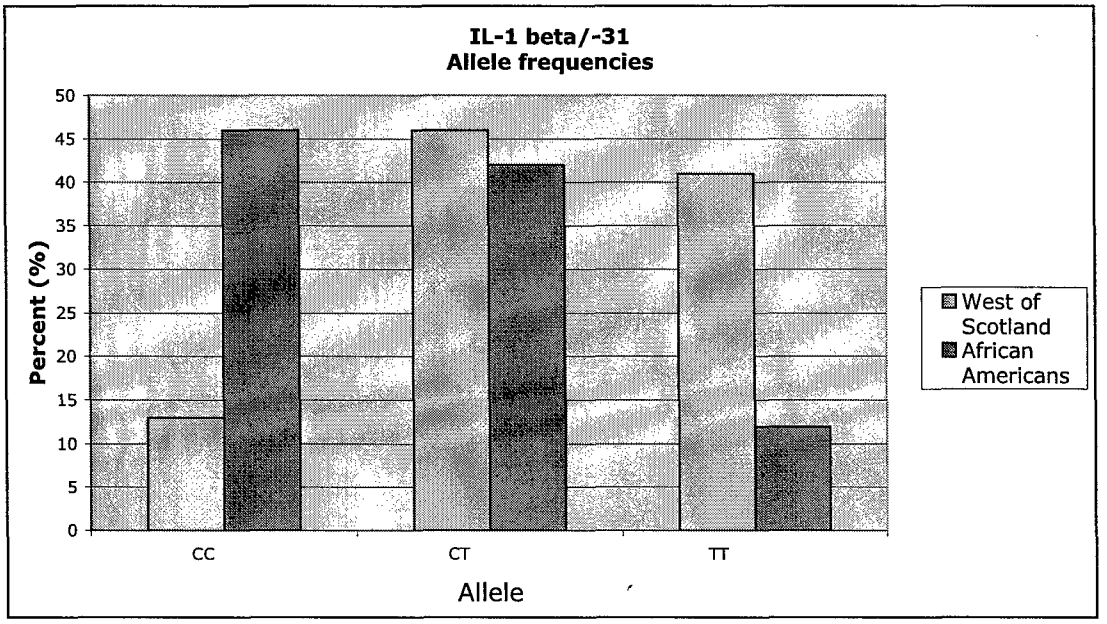


Chart 3

IL-1β/5810

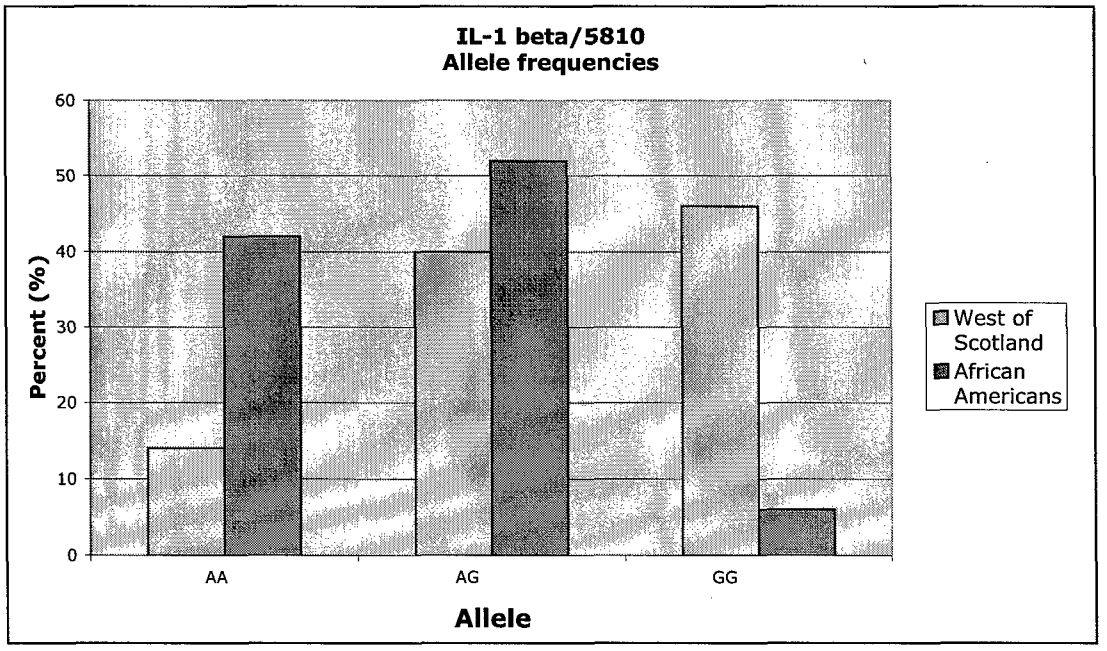


Chart 4

Therefore , these data demonstrate clearly that significant differences in allelic distribution exist between Caucasians and African Americans at two genetic loci within the IL-1 gene: IL-1 +5801 and IL-1 -511. The functional nature of these differences will be explored in future funding periods.

SECTION THREE

Evaluation of the Presence of Antibodies in the Oral Cavity of Mice Infected With Tularemia

The goals of this aim were to determine if mice that had deliberately been exposed to tularensis via aerosol exposure would develop immune abnormalities, which could be detected in either the oral lavage or in extracts obtained from periodontal scrapings. Tularemia is a rare but highly pathogenic disease caused by the small gram-negative coccobacillus, *Francisella tularensis.* This intracellular bacterial pathogen naturally causes tularemia in humans as well as other mammals, including rodents. Tularensis is one of the most infectious pathogenic bacteria known. It requires inoculation or inhalation of as few as 1-10 organisms to cause the disease. *F. tularensis* is found in widely diverse animal hosts and habitats and can be recovered from contaminated water, soil, and vegetation. Aerosol release of *F. tularensis* has the greatest adverse medical and public health consequences and greatest potential as a biological weapon.

F. tularensis can infect humans through the skin, lungs, gastrointestinal tract, and mucous membranes. It is a facultative intracellular bacterium that multiplies within macrophages. As with any infection, the immune system responds to the foreign antigen via the production of antibodies by B cells. Based on results obtained in year 01 of this BAA, the microarray analysis revealed that many of the genes activated in response *to F. tularensis* infection also were activated by lipopolysaccharide obtained from *E coli* (LPS). LPS is a known polyclonal B cell activator. One of the results of polyclonal B cell activation is an increase in the amount of "natural antibodies" as well as an increase in autoantibodies. Some of the deleterious effects that are a sequalae to several bacterial infections include arthritis and carditis. In this study, the presence of antibodies in the oral cavities of mice infected with tularemia was evaluated. Periodontal fluids and cell lysates were examined using an ELISA assay. CD14 knockout mice were also be used to determine if macrophage activation plays a role in the multiplication of the bacteria and subsequent antibody production.

Methods and Materials: *ELISA:*

In order to detect the presence of antibodies in the periodontal fluids and lysed cell suspensions obtained from tularensis infected and uninfected mice, an ELISA assay was performed. Briefly, 96 well plates are precoated for at least three hours with MBSA or coating solution. Plates were washed three times with 2% PBST and once with distilled water, and then 100ul of coating protein added which included: anti-IgM (to detect and quantitate IgM levels), ssDNA (to detect anti-ssDNA antibodies often associated with arthritis), or myosin (to detect anti-myosin antibodies often associated with carditis). Plates were incubated at 4°C for at least 24 hours. After the incubation, plates were blocked with 10x BSA blocking solution for 10 minutes and washed. At this time, 100ul of PBS (negative control), a standard (positive control), or sample (either oral lavage or periodontal cell lysate) was added to the wells and incubated at room temperature for one hour, washed and appropriate anti-mouse Ig-peroxidase conjugated antibodies

added and incubated for one hour at room temperature, washed and developed with 100ul of TBM HRP substrate followed by incubation for 20 minutes and then 100ul of H_2PO_4 is added to stop the reaction and the absorbance of the plates was read in a spectrophotometer at 450nm.

Results: From the ELISA assays, the amount of antibodies present in the mouse samples can be evaluated. *Figure 1.* compares the amount of antibodies found in tularensis infected versus uninfected mice. In the left panel, the plate was coated with glycerated goat anti-mouse u-chain specific IgM (Sigma M-8644). The right panel is coated with single stranded DNA from a calf thymus. Periodontal fluid from the sample mice was then placed in the wells at a 1:10 dilution. There was nearly a 2-fold increase in the overall level of IgM antibodies in the tularensis infected animals. In addition, there was a larger fold change in the amount of autoantibodies induced in response to tularensis infection. There is a large body of evidence that suggest that bacterial DNA not only serves as a specific antigen (bound to the BCR) but also bacterial DNA is able to serve as a polyclonal B cell activator via the presence of CpG motifs which bind to toll-like receptors (TLR). Oral lavage cell lysates were similar in that tularensis infected lysates had elevated IgM and ssDNA antibody production. CD14KO tularensis infected mice had higher levels of IgM than did the wild type infected mice, but this was not significant (data not shown).

Figure 1

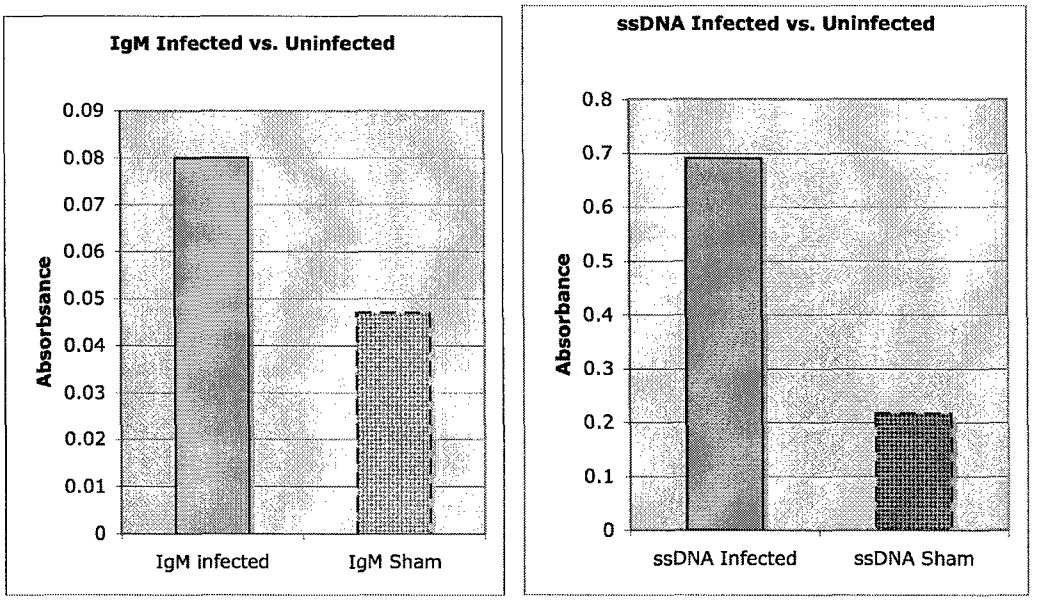


Fig 1. Antibodies present in perio fluid following Tularensis infection. Solid bars are infected animals and patterned columns are sham infected. The amount of IgM antibodies (left panel) and anti-ssDNA autoantibodies (right panel) were detected via an ELISA. Columns represent mean OD readings for each group (at least 4 mice per group)

A well-known example of a bacterial infection inducing autoimmune damage occurs following infection with Streptococcal . In this case the outer M protein contains epitopes that cross react with cardiac myosin. In the present study, the ability of tularensis infection to induce autoimmunity was further assessed via an ELISA assay to detect

anti-myosin reactivity. Myosin was used as a coating antibody (Myosin from Bovine Muscle, Sigma M0531 at 1 mg/ml). In *Figure 2*, the left panel compares the absorbance at 450nm of infected vs. uninfected samples from two separate experiments where the plates are coated with myosin and developed with the same secondary antibody that was used in the previous experiment. When the two separate experiments are averaged, a 2.5 fold increase in absorbance was observed in mice infected with tularensis.

Figure 2

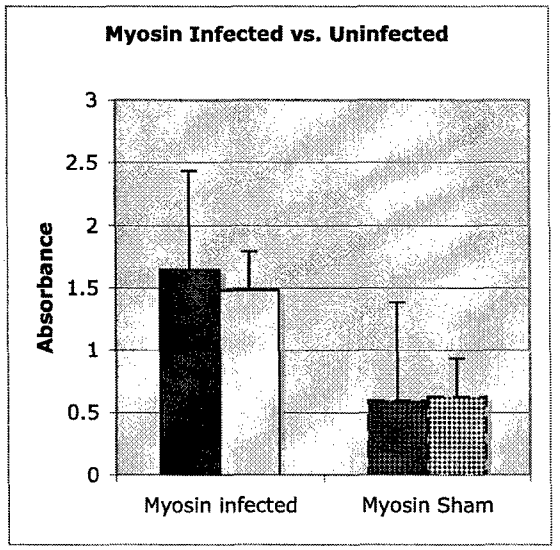


Fig 2. Levels of anti-myosin autoantibodies in periodontal fluid following Tularensis infection. Columns represent mean OD readings with the background subtracted from two separate experiments. In the left solid columns are the mean OD of the oral fluid obtain from infected mice and the right-hatched columns are from sham treated.

Discussion: We have begun to detect tularensis specific antibodies and preliminary data is that anti-tularensis antibodies are increased in mice previously infected with tularensis. However, we have used two sources of tularensis antigen and more work is required to optimize the assay. Based on the above results from the ELISA assays, it was determined that an increase in the presence of IgM antibodies could be seen in mice infected with tularensis. However, only an increase in the presence of IgM could be seen when comparing oral fluids from infected and uninfected mice. Additional samples of periodontal fluids and cell lysates analyzed on plates coated with goat anti-mouse IgG heavy and light chain (Caltag 2000) and rat monoclonal antibody to mouse IgG-2a (Caltag RMG2a00) and developed with a peroxidase labeled sheep anti-mouse IgG2a (The Binding Site, Code AP274) showed no significant presence of IgG in either infected or uninfected mouse samples despite the fact that these coating and secondary antibodies detected IgG in the NZB mouse sera which was used as a positive control (data not shown). Also contrary to expectations, no pattern of significant difference could be observed when comparing CD14 KO to wild type tularensis infected mice.

In order to improve upon these experiments, the next step would be to obtain an antigen found in tularensis (specifically an outer surface protein) that can be used to coat the plates for the ELISA assay.

SECTION FOUR – APPENDIX

As a preliminary exercise to determine the ability of the defensin LL-37 to kill select agents, we conducted preliminary experiments inthis area. In the following manuscript, currently being prepared for publication, we describe those initial data and show that LL-37 does indeed kill *B.anthracis*.

Introduction

Bacillus anthracis, the causative agent of anthrax, is a gram positive, spore forming bacterium found ubiquitously in nature. Anthrax disease manifests itself in one of three forms; cutaneous, gastrointestinal, and inhalational. Cutaneous anthrax occurrs due to contact with an infected sheep or other hooved animal. Gastrointestinal anthrax rarely happens, but can be contracted after eating contaminated foods. Inhalational anthrax occurs after a deliberate production and spread of finely milled spores in the air (Mock M 2001). Symptoms are vague, diagnosis is difficult, and even with antibiotic treatment it causes high mortality. It is for these reasons that *B. anthracis* is considered to be a highly effective weapon of mass destruction and an extremely potent and dangerous biological weapon (Inglesby TV 1999). As there is concern that organisms used in bioterror attacks will be resistant to one or more antibiotics, new antibiotics that are rapid and effective with limited side effects need to be found. Antimicrobial peptides (AMP) could be a viable alternative treatment.

AMPs display a range of structures that fall into four known classes: beta-sheet peptides (defensins), alpha helices (cathelicidins), extended helices and loop peptides. Cathelicidins are a group of cationic covalently unconstrained peptides that fold into amphipatic alpha helices. They derive from prepropeptides that share a highly conserved 100 amino acid N-terminal propeptide sequence. The C-terminus, which encodes the mature peptide, shows great diversity and is responsible for the differential antimicrobial effects. They are found in many mammalian species, including humans, cattle, sheep, rabbits, mice, and pigs (Phadke 2004).

The only cathelicidin identified in humans to date, LL-37 (hLL-37), consists of the linear C terminus of the human CAP-18 molecule. It has been identified in the epidermis, gastrointestinal tract and airway surface layer of the respiratory tract. hLL-37 has been shown to provide an immediate defense against infectious organisms. Over expression in lung tissues or application to a skin wound significantly decreases bacterial colonization. While LL37 exhibits direct antimicrobial activity, most likely due to disruption of the lipid bilayer (Henzler Wildman KA 2004) it also exerts many powerful and pleiotropic effects on both the innate and adaptive immune responses. Neutrophils constitutively express and store the peptide in granules, while it is an inducible product of epithelial cells, T cells and monocytes in response to infection (Bowdish 2004). Once released, hLL-37 exhibits chemotactic effects on monocytes, neutrophils, mast cells and T cells, thus attracting the immune cells to the site of infection (Oppenheim 2003).

The effect of LL-37 on *B. anthracis* has not yet been studied. *B.anthracis* possess two virulence plasmids, pX01 and pX02. pX01 encodes the tripartite exotoxin, which induces diverse effects including skin edema and cell death. pX02 encodes enzymes that control the formation of the glutamic acid capsule (Mock M 2001), which inhibits phagocytosis of the bacterium and induces septicemia. Strains missing one or both of these plasmids tend to have lower virulence and pathogenicity than the wild type (Fouet A 1996).

OBJECTIVE: To examine the bactericidal effects of two cathelicidins, hmLL-37 (Hylobates Moloch primate) and hLL-37 (human) on virulent (V1B) and avirulent (Sterne) strains of *B. anthracis*

MATERIALS AND METHODS

Bacterial Strains and Media: B*acillus anthracis* strains Vollum 1B (V1B) (pXO1+pXO2+) and Sterne (pXO1+pXO2-) were obtained from the United States Army Medical Research Institute of Infectious Disease (Fort Detrick, MD), in accordance with the Select Agent Regulatory requirements in 42CFR73. Overnight cultures were grown in Brain Heart Infusion Broth (Sigma, St. Louis, MO), and washed with 20% Tryptic Soy Broth (Sigma) before use in all assays with LL37. 20% Tryptic Soy Broth (TSB) (vol/vol) in Phosphate buffer (10mM pH7.4)(Sigma) was used as the growth medium for *B.anthracis* in the LL-37 assays due to the peptide's susceptibility to salt. *E.coli* 9634 (ATCC) was used as a control. Overnight cultures were grown in Luria-Bertani Broth (Sigma) and were washed with 20% TSB before use in the experiments. CFU were obtained by plating *B.anthracis* on NBY agar, while the *E.coli* was plated on LB agar, both sets of plates were incubated overnight at 37°C, 5% CO2.

Peptide synthesis: Peptides hmLL-37 (Hylobates Moloch primate) and hLL-37 (human) were kindly provided by Michele Boniotto and Sergio Crovella (University of Trieste).

Antimicrobial Susceptibilty Test to Determine Antibiotic Susceptibilty of *Bacillus anthracis:* Overnight cultures of *B.anthracis* V1B and Sterne were grown to mid log phase (OD600 0.5-0.8). 500ul of each culture was plated onto a 150x30 petri plate of Mueller Hinton agar. Antibiotic disks (Becton Dickinson, BBL Sensi-disc), Gentamycin 10ug, Ciprofloxacin 5ug, Chloramphenicol 30ug, Doxycycline 30ug, Streptomycin 10ug, were placed on the agar surface with sterile forceps. The plates were incubated overnight until zones of inhibition were visible. The growth inhibition zones were measured and the susceptibility or resistance was determined according to manufacturer's instructions.

Determination of Minimum Inhibitory Concentration (MIC) of Bacillus anthracis to gentamycin: Overnight cultures of V1B and Sterne were diluted 1/20 and allowed to incubate for 2hours at 37C with 5%CO2, to ensure the presence of exponentially growing cells. The cultures were then diluted to an OD600 of 0.05 (5x105 cfu/ml) and washed and resuspended in 20% TSB. Bacteria were added to each well containing various concentrations (5, 2.5, 1, 0.5, 0.25ug/ml) of gentamycin+ 20% TSB. After a 24-hour incubation at 37C, aliquots were taken from each well, serially diluted and plated to determine colony-forming units.

Treatment of *Bacillus anthracis* with LL-37: To ensure exponentially growing cells cultures of V1B, Sterne and *E.coli* were grown overnight at 37°C, then diluted to an OD600 of 0.1 and incubated for an additional 2 hours. hLL-37 and hmLL-37 were diluted in distilled water and filtered, to obtain a stock solution of 600uM.

All assays were performed in 96 well plates. Peptides were serially diluted in TSB with final concentrations (in uM); 100, 50, 25, 12.5, 6.25. 0.5ug/ml gentamycin, was used as a negative control. Bacteria were added to get a final concentration of 2.5x105 cfu/ml per well. A small aliquot of the bacterial suspension was sampled to control for cellular concentration. After 2 hours of incubation at 37°C, an aliquot of each well was serially diluted and plated onto NBY or LB agar. The plates were incubated overnight at 37°C, and colonies were enumerated the following day (performed in quadruplicate). To determine the time at which growth inhibition first occured, the same experiment as detailed above was performed but small aliquots were taken at 0, 15, 30, 60, 90, and

120 minutes, serially diluted and plated to determine colony forming units (performed in triplicate).
P values were generated using unpaired, 2-tailed Student's t test.

RESULTS

Disk Diffusion Assay and Gentamycin MIC

The agar disk diffusion test was performed in order to determine the most effective antibiotic against these strains of Bacillus anthracis that could be used as a positive growth inhibitor control in the LL-37 experiments. Gentamycin provided the best growth inhibition zone in this experiment and was chosen as a positive inhibition control. In order to determine the appropriate concentration of gentamycin to use in the LL-37 experiments, the minimum inhibitory concentration experiment was performed. It was determined that 0.5ug/ml of gentamycin was sufficient.

Sterne is more susceptible than V1B to growth inhibition by both hLL-37 and hmLL-37

V1B and Sterne, although being two different strains belonging to the same species, exhibited different behavior when treated with antimicrobial peptides. Lower concentrations of each peptide were needed to inhibit growth of Sterne than of V1B (a reduction of at least 3 log10 CFU/ml, marked by the arrows in Figure 1). 100uM concentrations of both hLL-37 and hmLL-37 were required to achieve this level of growth inhibition of V1B. Colony forming units of Sterne however, were reduced 3 log10 with only 25μM of hLL-37 and 50μM of hmLL-37.

The greater susceptibility of Sterne to LL-37 is further observed when comparing growth of LL-37 treated groups to untreated bacteria. For Sterne the lowest concentration of hLL-37 tested, 6.25μM, caused a statistically significant reduction in CFU from the untreated group. A slightly higher concentration of hmLL-37, 12.5μM, yielded a statistically significant decrease of Sterne CFU counts from untreated organisms. For V1B, however, twice the concentration of each peptide 12.5μM hLL-37 and 25μM hmLL-37 was needed to achieve CFU counts significantly less than untreated bacteria.

hLL-37 has greater bactericidal effect than hmLL-37 on Sterne but not on V1B

The data shown in Figure 1 suggests that there are varying levels of susceptibility of each strain to LL-37 treatment. It is equally important to investigate the potential differences in antimicrobial activities of each peptide (Figure 2).

When measuring growth inhibition of Sterne, hLL-37 demonstrated significantly greater activity than hmLL-37 at all concentrations tested but 100uM (Figure 2A, * $p < 0.05$). However, no significant differences in bactericidal effect against V1B were observed between the 2 peptides (Figure 2B).

hLL-37 starts inhibiting the growth of both V1B and Sterne faster than hmLL-37

To explore the differences in the bactericidal activity of each peptide a time course assay was performed to look for possible variations in the kinetics of killing. Growth inhibition of both Sterne and V1B was first observed after only 15 min incubation with 100uM of hLL-37 (Figure 3, panels A and B). Not surprisingly, it took 60 minutes for both strains to exhibit growth inhibition in the presence of 100μM hmLL-37 (Figure 3, panels C and D). Again, the greater susceptibility of Sterne to LL-37 treatment was noticed throughout the time course experiments. Complete killing of Sterne was achieved in 60 minutes in the presence of 100μM concentration of hLL-37 and 120 minutes with the same amount of hmLL37. In contrast, V1B cultures were not abolished by either peptide.

DISCUSSION

In recent years, specifically after the 2001 anthrax scare, *B. anthracis* has shown its ability to be an easy, effective, and potent weapon. Since LL-37 is known to have antimicrobial effects, it's use as an alternative to antibiotic treatment shows promise. The data contained herein highlights differential effects of two cathelicidins, the human hLL-37 and the primate hmLL-37, on two different strains of *B. anthracis*, as an approach to screen for AMP that could be used as an alternative treatment for anthrax infection.

Overall, the results suggest that Sterne is more susceptible than V1B to both hLL-37 and hmLL-37, as higher amounts of each of these peptides were needed to inhibit the growth of V1B the same extent as Sterne, as shown in figure 1. When comparing the ability of the peptides to inhibit growth (Figure 2), hLL-37 had greater bactericidal effect on Sterne than hmLL-37. However V1B was affected likewise by both peptides at all concentrations tested.

Time course experiments were then performed to determine the time at which growth inhibition commenced (Figure 3). At 100uM hLL-37 starts inhibiting the growth of both V1B and Sterne before than hmLL-37 (15 and 60 minutes respectively) when compared to untreated bacteria. Also, at that concentration, hLL-37 reduced the counts of Sterne to zero in 60 minutes, while it took 120 minutes to achieve the same result with hmLL37. This supports our previous finding shown in Figure 2, that hLL-37 has greater bactericidal effect on Sterne than hmLL-37. Not surprisingly, neither peptide was able to decrease V1B counts to zero, confirming that Sterne is more susceptible than V1B to growth inhibition. Although there is a difference in the kinetics of V1B killing by hLL-37 and hmLL-37 (V1B is killed faster by hLL-37 than by hmLL-37), the final bacterial counts at the end of two the hour treatment are not significantly different as shown in Figure 2.

This evidence of differential activity of human and primate cathelicidins is not unexpected, as the antimicrobial effect of cathelicidins is strongly dependent upon their compositions. A difference in one amino acid is strong enough to render the cathelicidin inactive (Periathamby Antony RAJ 2000; Brogden 2003). Other reasons rather than variations in the peptides sequences could explain the differential susceptibilities of the attenuated and virulent strain to their bactericidal effects. It could be due to differences in the mechanisms of action of these two peptides and/or differences in the bacterial morphology of these two strains. Recently it has been shown that hLL-37 can act on the lipid bilayer, inserting into the hydrophobic region thus resulting in disruption of the cell membrane (Henzler Wildman KA 2004). Whether or not this interference is responsible for the antimicrobial effects on *B. anthracis* is not known. The peptides may kill the bacteria also through some other mode.

The varying susceptibilities of each strain to LL-37 may be also be partially explained by their morphological differences. The capsule present on V1B acts as an effective physical barrier and may interfere with the penetration of the peptide, thus providing some resistance to its antimicrobial effects. Since Sterne lacks this capsule, LL-37 may easier access to the cell membrane and into the cell. Future experiments include the disruption the capsule of V1B prior to LL-37 exposure, thus determining if the capsule is indeed responsible for the enhanced resistance to their bactericidal effects. Other possible phenotipical differences between strains such as protein secretion will also be investigated. It has been shown that Sterne and V1B secrete a different pattern of proteases. V1B could be secreting a protein able to degrade LL-37. Assays need to be perfomed in order to detect extracellular protease activitiy in supernatants of V1B and Sterne cultures. Also, supernatant swap experiments will be carried out to determine if extracellular proteins secreted by V1B help protect Sterne from LL-37.

hLL-37 has been shown to play an integral role in the human innate and adaptive immune response. Addition of hLL-37 to human dendritic cells before infection with

anthrax up regulates the immune response of these cells, inducing them respond more rapidly to the pathogen (Donald J. Davidson 2004).

The results of these experiments provide many interesting avenues to explore in order to gain a fuller knowledge of the antimicrobial effects of LL-37 on anthrax. Further screening of previously uninvestigated cathelicidins will undoubtedly unearth additional effective antimicrobial peptides. Combinations of LL-37 with different effective antibiotics could be then performed to try to enhance the bactericidal effect.

Investigating into the different mechanism of inhibition between cathelicidins will allow for the development of an alternative treatment for anthrax that is effective, rapid and complements the natural immune response.

Bowdish, D. M., Davidson, D.J., Speert, D.P., Hancock, R.E. (2004). "The human cationic peptide LL-37 induces activation of th extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes." Journal of Immunology **172**(3758-65).

Brogden, K. A., Mark Ackermann, Paul B. McCray, Jr., Brian F. Tack (2003). "Antimicrobial peptides in animals and their role in host defences." International Journal of Antimicrobial Agent **22**: 465-478.

Donald J. Davidson, A. J. C., Gregor S.D. Reid, Dawn M.E. Bowdish, Kelly L. MacDonald, Rebecca C. Ma, Robert E.W. Hancock, and David P. Speert (2004). "The cationic antimicrobial peptide LL-37 Modulates Dendritic cell differentiation and Dendritic cell-induced T Cell Polarization." The Journal of Immunology **172**: 1146-1156.

Fouet A, M. M. (1996). "Differential Influence of the Two *Bacillus anthracis* Plasmids on Regulation of Virulence Gene Expression." Infection and Immunity **64**(12): 4928-4932.

Henzler Wildman KA, M., G.V., Brown, M.F., Ramamoorthy A. (2004). "Perturbation of the hydrophobic core of lipid bilayers by the human antimicrobial peptide LL-37." Biochemistry **43**: 8459-69.

Inglesby TV, H. D., Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Hauer J, McDade J, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Tonat K. (1999). "Anthrax as a biological weapon: medical and public health management. Working Group on Civilian Biodefense." JAMA **281**(18): 1735-45.

Mock M, F. A. (2001). "Anthrax." Annu. Rev. Microbiol. **55**: 647-671.

Oppenheim, J. J., A. Biragyn, L.W. Kwak, D. Yang (2003). "Roles of antimicrobial peptides such as defensins in innate and adaptive immunity." Ann. Rheum. Dis. **62**((supplement II)): ii17-ii21.

Periathamby Antony RAJ, K. J. A. a. T. K. (2000). "Large Scale synthesis and Functional elements for the antimicrobial activity of defensins." Biochemistry Journal **347**: 633-641.

Phadke, S. M. (2004). "Defensins and Other Peptides." Pediatric Pulmonology, Supplement **26**: 74-76.

FIGURES

FIGURE 1

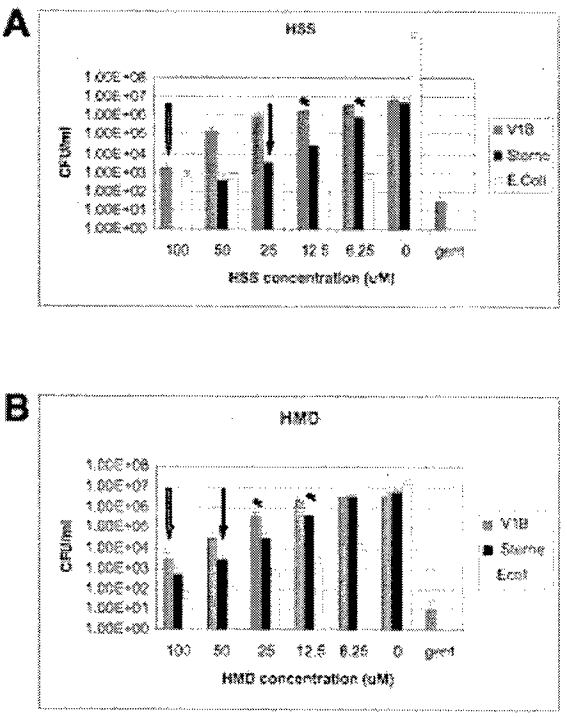
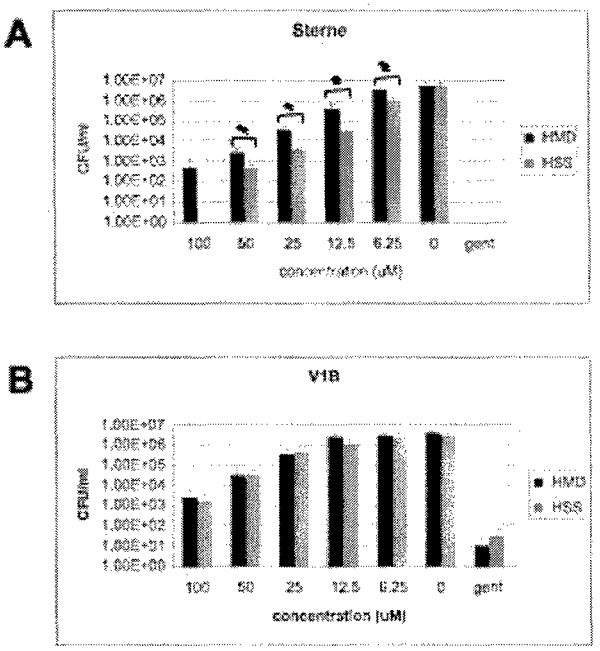


FIGURE 2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CFU/ml
100
50
25
12.5
6.25
0
gent
concentration (uM)
HMD
HSS
B
V1B
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1.00E+03
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1.00E+01
1.00E+00
CFU/ml
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50
25
12.5
6.25
0
gent
concentration (uM)
HMD
HSS

FIGURE 3